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SCREENING OF CAVE BACTERIA FOR ANTIMICROBIAL ACTIVITY AGAINST *PSEUDOMONAS AERUGINOSA* BIOFILMS

2015 | COHORD MASON

B.Sc. Honours thesis

THOMPSON RIVERS UNIVERSITY

Screening of Cave Bacteria for Antimicrobial Activity against *Pseudomonas aeruginosa* Biofilms

by

COHORD MASON

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ABSTRACT

The bacterium *Pseudomonas aeruginosa* may grow in a biofilm structure, which can be up to 1000 times more resistant to antibiotics compared to planktonic isolates. The *P. aeruginosa* biofilms have serious implications with regard to infection, especially in individuals with weakened immune defenses such as burn and cystic fibrosis patients. Antibiotic studies are usually based on planktonic antibiotic susceptibility results, so the treatment may be less effective when used in patients. The aim of this study was to further screen previously studied cave bacterial isolates with potential activity and determine their antimicrobial capabilities against *P. aeruginosa* biofilms. Three strains; A1A3, RA003 and 58B were cultured in different media over 10 days, with collections of supernatant on days 2, 4, 6, 8, and 10. The MBEC P&G Assay device was used to culture *P. aeruginosa* biofilms, which were then exposed to the collected supernatants. After exposure, the surviving biofilms were recovered, and spot plated in order to measure any inhibition of *P. aeruginosa*. Dilution and spot plating were also used to enumerate surviving cells, and give a percent survival quantification of antimicrobial activity. As well, Kirby-Bauer disc diffusion assays were used to determine the cave isolates' inhibitory effect on planktonic cells. Scanning electron microscopy was used to examine biofilms structures and 16S rRNA sequencing was used to identify cave bacterial isolates. The biofilms showed a noticeably decreased percent survival when exposed to the cave isolate supernatant. The cave isolate 58B showed to be very promising, demonstrating significant reduction in the surviving biofilm cells. This study shows that cave bacteria produce antimicrobials that are effective against pathogenic bacteria even in a biofilm structure.

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Contents

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Antibiotic Resistance

The decline in the discovery of novel antibiotics and rising of antibiotic resistance in pathogens has made the discovery of new ones important. Antibiotics are essential to the medical field, and help us combat bacterial and fungal infections, which, according to the World Health Organization, cause 16.2% of the world's deaths per year. The antibiotics work by interacting with a bacterium or fungi, and disrupting a certain pathway resulting in the organism's destruction. When the antibiotics are first introduced, there may be a small proportion of resistant bacteria in patients, but these bacteria are not yet problematic. Then the number of resistant bactereria may rise to a level where the antibiotic has reduced efficiency in the human population, resulting in antibiotic resistant bacteria (Coates and Hu, 2007). Antibiotic resistance precedes clinical use, and can be found in nature (Hall and Barlow, 2004), but has increased due to the over-use in bacterial infection therapies (Fernández et al., 2011). From the overuse of antibiotics, some bacteria have seemingly undergone an accelerated evolution, and have increased success of surviving antibiotic treatment. This has allowed some bacterial species to become "multi-drug resistant" (MDR). Through continuous exposure, some bacteria have become resistant to these compounds and employ many different defenses. These defenses may be passed through horizontal gene transfer, conjugation, or mutations leading to decreased susceptibility (Baquero, 2001). Antibiotic resistance may be from bacteria having enzymes that break down the antibiotic, reducing the permeability of the bacterial cell, or from changing the target of the antibiotic.

An interesting example of a resistant defense is biofilms that are produced by some bacterial species.

Biofilms

Biofilms are communities of bacteria encased in a self-synthesized polymeric matrix that may adhere to biotic or abiotic surfaces (Hall-Stoodley et al., 2004). The cells of the biofilm are typically encased in a matrix of polysaccharides, extracellular DNA (eDNA), and proteins (Flemming and Wingender, 2010; Yang et al., 2012). Bacteria are usually thought of as planktonic, single cell organisms, but they predominantly live in multicellular biofilms in the environment. Environmental stress results in dysregulation of multiple regulatory systems, and the cells will attach to a surface in order to resist the environmental change. The formed mature biofilm may release some cells in a dispersive stage in order to colonize new areas (Costerton et al., 1999; Hall-Stoodley et al., 2004).

Biofilms may have water-filled channels that allow for increased nutrient supply. The matrix may also contain extracellular enzymes, and cells may show enhanced toxic compound excretion, changed metabolic processes and differentiated phenotypes. That is, the biofilm creates a gradient of nutrients and oxygen with inner cells having a decreased metabolism and cell division relative to the peripheral cells (Costerton et al., 1999; Flemming and Wingender, 2010; Hall-Stoodley et al., 2004). This mode of growth protects the bacteria from eradication via desiccation, nutrient deprivation and antibiotic treatment (Gaddy and Actis, 2009). Bacteria associated with biofilms may be up to 1000 times more resistant to antibiotics compared to planktonic cells, and enables cells in biofilms to persist despite intensive antibiotic therapy (Gaddy and Actis, 2009; Mah and O'Toole, 2001). Antibiotic

therapy may even trigger biofilm formation if the antibiotics are at a concentration below the minimum inhibitory concentration (known as sub-MIC). Bacteria may be exposed to sub-MIC at the beginning and end of antibiotic therapy. Also, the colony of cells may be thick enough such that only low amounts of the antibiotic actually make it to inner cells, resulting in sub-MIC (Mah and O'Toole, 2001; Singh et al., 2010). The bacterium *Pseudomonas aeruginosa* is a clinically important organism because it forms resilient biofilms.

Pseudomonas aeruginosa Pathogen

Pseudomonas aeruginosa is a motile, Gram-negative, opportunistic pathogen that is prevalent in the medical hospital environment. The pathogen is common in respiratory infections and urinary tract infections, and is especially implicated in patients with weakened defenses like burn and cystic fibrosis (CF) patients (Morita et al., 2014). The bacterium *P. aeruginosa* is a metabolically versatile microbe that can survive in many different environments. It can grow in aerobic and anaerobic conditions and may produce a multitude of different virulence factors (Schurek et al., 2012). The pathogenic bacteria may possess flagella and type IV pili that can function in adhesion and motility as well as initiation of an inflammatory response (Gellatly and Hancock, 2013). Also, Type 3 secretion systems inject toxins directly into host cells and breach epithelial cell layers (Hauser, 2009). The bacterium *P. aeruginosa* may also produce proteases that are able to degrade immunoglobulins and fibrin, leading to tissue damage (Kipnis et al., 2006). Some other virulence factors include exotoxin A, lipases, pyocyanin, and iron chelators (Gellatly and Hancock, 2013). As well as possessing many virulence factors, *P. aeruginosa* is also resistant to many antibiotics. The bacterium has intrinsic resistance to many antimicrobials because of an outer-membrane barrier, multidrug efflux pumps and endogenous antimicrobial inactivation (Poole, 2011).

Most importantly the bacterium can produce a robust biofilm, which is thought to further its antibiotic resistance ability. This resistance by *P. aeruginosa* has allowed it to persist despite tough antibiotic therapies so there is a need for new antibiotics.

Cave Bacteria

Caves are extreme habits that may house new microorganisms, which can potentially produce many new bioactive compounds. The caves are usually nutrient deprived so these organisms must have adapted in order to survive in such an environment (Cheeptham et al., 2013). In particular, Gram-positive, filamentous actinomycetes have been proven to be a new source of antibiotics (Genilloud et al., 2011). Out of 22,500 biologically active compounds, 45% are from actinomycetes (Bérdy, 2005), and the species *Streptomyces* accounts for 70% of the total antibiotic production (Lam, 2006). Cave actinomycetes are of particular interest because of the unique environment that they live in. It is reasonable to hypothesize that microorganisms living in caves are subject to regressive evolution, where some non-essential traits are lost over time. The extreme environment would result in bacteria losing some genes but developing new ones with different metabolic pathways. These new pathways could also result in new secondary metabolites, which could be important in drug discovery (Cheeptham et al., 2013).

Activation of Antimicrobial Compounds with Ultraviolet Light

There have been many studies on the activation of natural compounds with either full spectrum sunlight or ultraviolet light. These studies include the activation of biocidal compounds such as plant extracts, cow urine and even some antibiotics (Cheeptham and Towers, 2002; Upadhyay et al., 2010; Yuan et al., 2011). In most cases, organic compounds

absorb UV light and results in photolytic degradation, but this can be gradual which may result in new conformations of the compound (Kim and Tanaka, 2009). In one such study UV was used on antibiotics like ciprofloxacin, which resulted in an increase of activity against to *Vibrio fisheri* (Yuan et al., 2011). Our study also used UV light to potentially activate natural compounds to determine if they have any increased antimicrobial properties against a biofilm.

Objective

The objective of this research was to test the hypothesis that secondary metabolites produced by cave actinomycetes will have antimicrobial and/or antibiofilm properties against *Pseudomonas aeruginosa* in a biofilm. This includes determining the most effective cave bacteria isolate against *P. aeruginosa*, as well as the most proficient growth conditions to produce that effective secondary metabolite. Three cave bacteria isolates (A1A3, RA003, and 58B) previously showing activity were cultured for 10 days in different fermentation media, with samples taken on each day throughout the fermentation period. Collected samples were used to treat *P. aeruginosa* biofilms using a 96 well plate assay. The antimicrobial activity was measured by using dilution series and viable cell counts. A Kirby-Bauer disc diffusion assay was used to determine the effect of the supernatant on planktonic cells. Scanning electron microscopy was used to examine the biofilms presence and structure. Antibiotic susceptibilities of biofilms and planktonic cells compared the difference in resistances to the antibiotics. Lastly 16s rRNA sequencing identified the cave isolates' genera.

MATERIALS AND METHODS

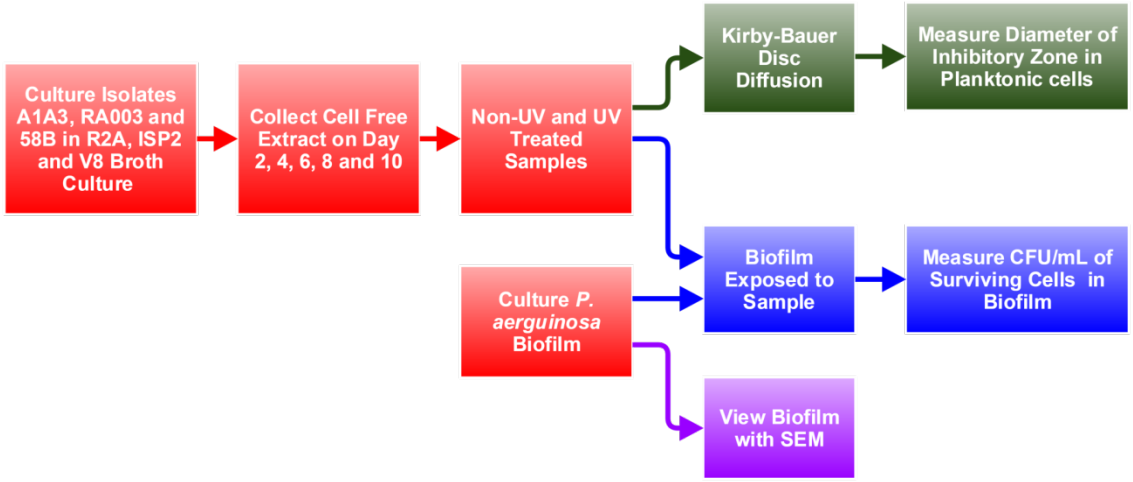

Figure 1: Flow diagram representing the sample collection, and exposure of *P. aeruginosa* to the cave bacterial supernatant samples.

Culturing of *Pseudomonas aeruginosa* Biofilms in MBEC Device

The MBEC P&G assay device was used to culture the *P. aeruginosa* in a biofilm formation according to Standard Test Method for Testing Disinfectant Efficiacy against *Pseudomonas aeruginosa* Biofilm using the MBEC Assay (2014). The pathogenic bacteria *P. aeruginosa* (lab strain with unknown strain number) was first subcultured onto a trypticase soy agar (TSA) (Appendix A) plate using the four way streak method for isolated colonies. The plate was incubated for 18 hours at 35^{o}C, and then inspected after the incubation to ensure a pure culture.

Once the TSA plate had pure colonies of *P. aeruginosa*, the biofilm culturing began. 3.0 mL of sterile trypticase soy broth (TSB) was put into a sterile 16x100 mm glass test tube. A sterile cotton swab was used to collect *P. aeruginosa* colonies from the TSA plate. The swab with the bacteria was then deposited into the glass tube with TSB. The sample was

brought to a 1.0 McFarland standard using the deposited bacteria. This was achieved by taking a 500 uL sample and using a spectrophotometer at 600 nm (1.0 Mcfarland standard; OD=0.257 at 600 nm). Once the sample was at a 1.0 McFarland standard, 1.0 mL was added to 29 mL of TSB in a 45 mL plastic sterile centrifuge tube (this resulted in a 30x dilution) and served as the inoculum. A sterile MBEC P&G assay 96 well plate (Innovotech, Suite 101, 2011-94 St. Edmonton, AB, Canada) (Figure 2) was then removed from the packaging. All 96 wells were aseptically filled with 150 μL of the inoculum previously prepared. The MBEC device was then put in a 35ºC incubator with an orbital shaker at 100 rpm for 18 hours. After 18 hours, the MBEC device was removed from the incubator.

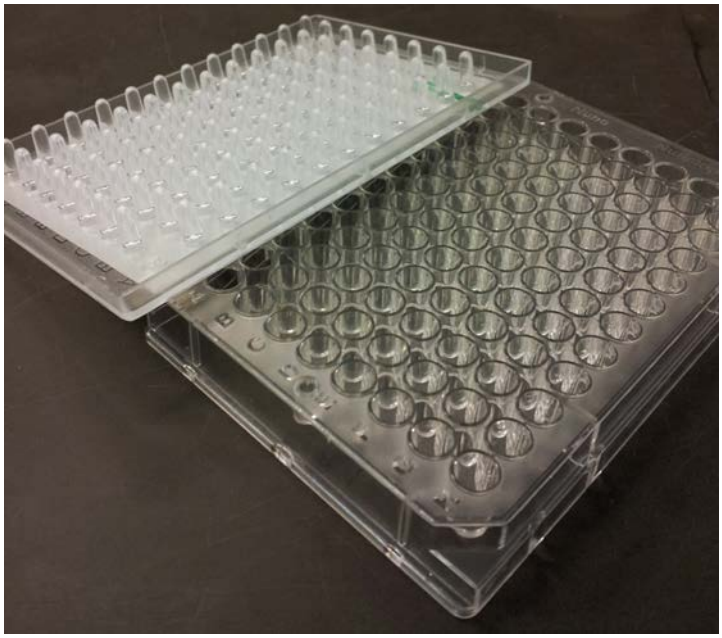

Figure 2: The MBEC P&G device used to culture *P. aeruginosa* into a biofilm formation. The device is composed of a 96 well plate with a matching 96 peg lid, with each peg fitting into a well.

Scanning Electron Microscopy Preparation and Set-Up: Viewing *Pseudomonas aeruginosa*

The pegs with the *P. aeruginosa* biofilms were then removed from the lid of the MBEC device. This was achieved by using sterilized (flamed) needle nose pliers, grasping the pegs closed to the lid, and breaking the peg from the lid. The pegs were then put in 0.9% saline solution for one minute to remove any planktonic cells. The pegs were then placed horizontally on a stub (Specimen mount, pin type, slotted head) with carbon tape, and were loaded into the chamber of the Zeiss LS EVO SEM. The operating conditions used were: the vacuum at EP 60 Pa, accelerating voltage at 20 kV, filament amperage 1.684 A, spot size 100 pA, working distance 12 mm and the VPSE G3 detector (personal communication with Dr. Cindy Ross Friedman). The resulting images were adjusted for brightness and contrast with Microsoft Word tools to achieve the best picture quality.

Culturing of Cave Bacteria Isolates

The screening of cave bacteria isolates used three different isolates that previously showed activity against *P. aeruginosa* in a biofilm formation (Mason, 2015). The three strains used were RA003, A1A3, and 58B because they showed the ability to inhibit biofilms. These samples originally came from a volcanic cave at Wells Gray Provincial Park in British Columbia. Some samples came from agar plates stored in a 4^{o}C fridge, and some from storage in 10% glycerol in 96 well plates at a temperature of -70^{o}C (cryopreservation). Samples on agar plates had colonies removed with a sterile loop, and deposited on fresh Hick-Tresner (HT) (Appendix A) agar plates. The deposited sample was streaked for isolated

colonies using the 4-way streak method in order to achieve pure colonies of the cave isolates. Samples form cryopreservation were allowed to thaw slightly to liquid, then 10 μL samples were removed, deposited on HT agar plated and streaked for confluent growth. These samples were then used to inoculate new HT agar plates, which were then streaked for isolated colonies in order to obtain pure cultures. These plates were incubated at 25ºC for 7 to 10 days and checked for pure colonies. Plates with mixed growth were then cultured again to obtain pure samples. Plates with pure growth were parafilmed and stored at 4ºC for later use. Colony morphology, Gram-staining and microscopy were used to confirm samples were isolates from the previous studies.

The three isolates were then used to inoculate test tubes containing different broth media. The 16X100 mm test tubes were filled with 6 mL of either V-8 juice, International Streptomyces Project #2 (ISP2), or R2A fermentation media (Appendix A), then autoclaved at 121ºC for 15 minutes. Then 0.5x0.5 cm square plugs were aseptically cut out of the agar plates containing colonies of the desired cave bacterial isolate. These plugs were then put in the appropriate test tubes and cultured at 25ºC for 10 days with 100 rpm of agitation in an orbital shaker (New Brunswick Scientific Innova 42).

The samples of the supernatant from each tube were taken at days 2, 4, 6, 8, and 10. The test tubes were removed from the incubator and put on a sterilized bench. Then 800 μL samples were removed from each tube with care taken not to gather any cellular debris present. The 800 μL samples were then put into 1.0 mL Eppendorf tubes and stored at -20ºC until used.

Antimicrobial Exposure

The MBEC device was used to perform antimicrobial exposures. The MBEC device was removed from the incubator; the peg lid was removed and rinsed once in 0.9% saline solution for 1 minute (Figure 3). The supernatant collected from the cave bacteria samples was thawed in the 1.0 mL Eppendorf tubes. The tubes were then put in a centrifuge at 5000 rpm for 2 minutes. Any remaining cell debris would then be forced to the bottom of the Eppendorf tube. A sterile Nunc 96 well plate (washed with 2% Virkon, 70% ethanol, then exposed for 20 minutes to UV light) was used for the antimicrobial exposure and deemed the "challenge plate." The wells for the UV treated samples were filled with 200 µL of the supernatant from the appropriate cave bacteria sample with triplicates of each. The 96 well plate with the supernatant was then exposed for 30 minutes to UV light (254 nm) in a Labconco Class II biological safety cabinet.

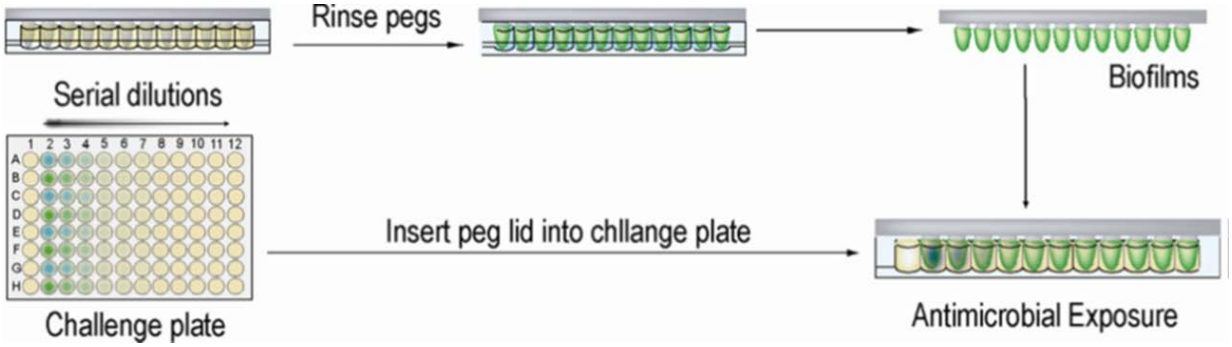

Figure 3: Diagram of rinsing procedure used for preparation for the initial removal, challenge plate and recovery plate (Ceri et al., 1999).

The Non-UV samples and controls were then added to the 96 well plates. The wells for the Non-UV treated samples were filled with 200 µL of the supernatant from the appropriate cave bacteria sample. The controls were then added to the appropriate wells: Sterile TSB, 2% Virkon, sterile V8, sterile R2A, sterile ISP2 and sterile 1.0 N HCl or water

(Figure 4). The growth controls would be those biofilms exposed to only sterile TSB, and the fermentation media controls would be biofilms exposed to the sterile V8, R2A, ISP2, and 0.1 N HCl or water. The Positive control was the biofilms that were exposed to a 2% Virkon solution (A disinfectant effective against *P. aeruginosa*), as well as the antibiotics Tetracycline HCl (120, 60, 30, and 10 µg/mL) and Ciprofloxacin in 0.1 N HCl (10, 5, 2.5 and 0.5 µg/mL) or Ciprofloxacin HCl (10, 5, 2.5 and 0.5 µg/mL).

	1	2	3	4	5	6	7	8	9	10	11	12
A	TETRA 120 µg/mL	TETRA 60 µg/mL	TETRA 30 µg/mL	TETRA 10 µg/mL	TETRA 120 µg/mL	TETRA 60 µg/mL	TETRA 30 µg/mL	TETRA 10 µg/mL	TETRA 120 µg/mL	TETRA 60 µg/mL	TETRA 30 µg/mL	TETRA 10 µg/mL
B	CIPRO 10 µg/mL	CIPRO 5 µg/mL	CIPRO 2.5 µg/mL	CIPRO 0.5 µg/mL	CIPRO 10 µg/mL	CIPRO 5 µg/mL	CIPRO 2.5 µg/mL	CIPRO 0.5 µg/mL	CIPRO 10 µg/mL	CIPRO 5 µg/mL	CIPRO 2.5 µg/mL	CIPRO 0.5 µg/mL
C	A1A3 R2A NON	A1A3 ISP2 NON	A1A3 V8 NON	R003 R2A NON	R003 ISP2 NON	R003 V8 NON	58B R2A NON	58B ISP2 NON	58B V8NON	R2A sterile control		
D										ISP2 sterile control		
E										V8 sterile control		
F	UV	UV	UV	UV	R2A UV	ISP2 UV	UV	UV	UV	Sterile TSB		
G										2% Virkon		
H										0.1 N HCl sterile		

Figure 4: Challenge plate schematic containing the 3 cave bacteria isolate samples with UV and Non-UV treatments cultured in R2A, ISP2, and V8 media. There are 2 antibiotics used in four concentrations: Tetracycline HCl at 120, 60, 30, and 10 µg/mL and Ciprofloxacin at 10, 5, 2.5, and 0.5 µg/mL. Also there is 2% Virkon controls, 0.1 N HCl (or sterile water) controls and sterile media controls (R2A, ISP2, and V8).

The peg lid from the MBEC device (after rinse [Figure 3]) was placed in the challenge plate with the appropriate orientation. The peg lid with the Challenge plate was then incubated at 35°C (no shaking) for 18 hours after which the MBEC device was removed from the incubator.

Neutralization of the Antimicrobial and Recovery of Surviving Bacteria

After the peg lid and challenge plate was removed from the incubator, the peg lid was rinsed twice in 0.9% saline solution for 1 minute each wash. A sterile Nunc 96 well plate had all 96 wells aseptically filled with 200 µL of D/E neutralizing broth (Appendix A). The peg lid was then placed in the D/E neutralizing broth for 1 hour to neutralize the antimicrobials. The peg lid was then placed in a sterile 96 well plate with all 96 wells filled with 200 µL of TSB (deemed "recovery plate"). The peg lid and recovery plate were then sonicated for 30 minutes to remove the biofilm from the pegs.

Two large plates (Figure 5), each filled with 250 mL of TSA, were used to check the results of the antimicrobial exposure. Each plate was divided into 49 sections, each with the appropriate sample area (Non-UV treated, UV treated, controls etc.). Then, 10 µL from each well of the recovery plate was spot plated onto the appropriately labelled square. The two TSA plates were then incubated at 35^{o}C for 18 hours, and the recovery plate was put in a -4^{o}C fridge.

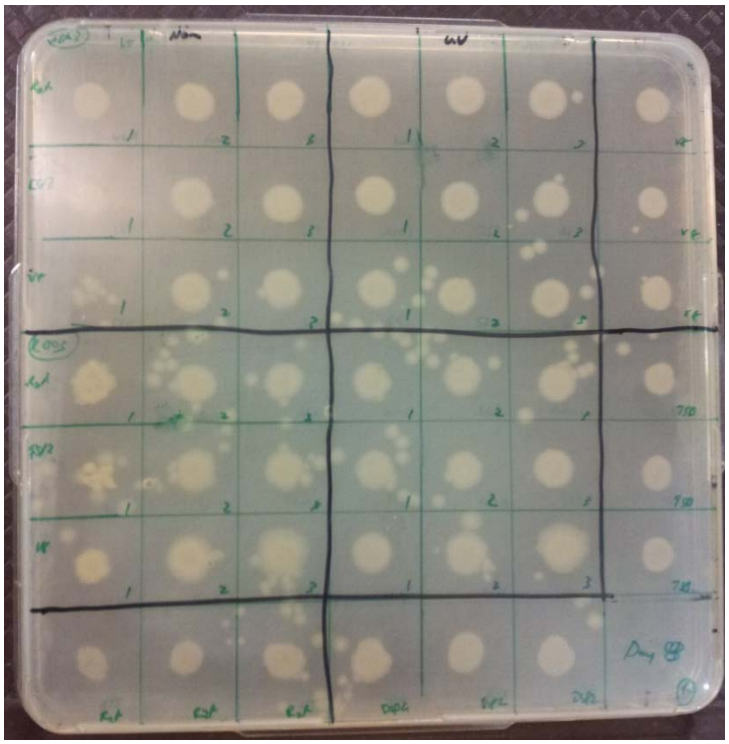

Figure 5: Example of Large TSA plates used to visually determine growth of *P. aeruginosa* (Picture after 18 hours of incubation in 35ºC).

The TSA plates after 18 hours were removed and visually inspected to determine if any samples had no growth or reduced growth. Any samples showing no growth or reduced growth were spot plated to enumerate the colonies and to determine the CFU/mL. The recovery plate with the same sample on the TSA plate was used to make 4 dilutions from 10^{-1} to 10^{-4}. The dilutions then had 10 uL removed and spot plated onto TSA plates. As well the *P. aeruginosa* growth controls (only exposed to TSB) and sterile media controls (R2A, ISP2, and V8) were also diluted the same and spot plated onto TSA plates. These TSA plates were then incubated at 35ºC for 18 hours.

After 18 hours, the TSA plates were removed from the incubator. The TSA plates had the cell colonies enumerated in order to determine the CFU/mL. The CFU/mL for each sample was then subjected to a 2 sample T test with pooled variances (after testing data for normality

and equal variance) to determine if the difference was significant. A P value of <0.05 was considered significant. A sample that showed 0 CFU/mL of surviving bacteria was designated bactericidal or if the concentration was known, then it was the minimum biofilm eradication concentration (MBEC).

Kirby-Bauer Disc Diffusion Assay to Determine Planktonic Antimicrobial Activity

The standard Kirby-Bauer (KB) disc diffusion assay (Bauer et al., 1966; Wilkins et al., 1972) was used to determine the antimicrobial activity of the cave isolates against planktonic *P. aeruginosa*. The discs used were 8 mm Advantec paper discs (Tokyo, Japan) that had previously been autoclaved to sterilize them. Each cave bacterial isolate sample was put on the discs in replicates of three. The antibiotics tetracycline HCl (120, 60, 30, and 10 μg/mL) and ciprofloxacin (10, 5, 2.5 and 0.5 μg/mL) or ciprofloxacin HCl (10, 5, 2.5 and 0.5 μg/mL) as well as 2% Virkon were used as positive controls. Additionally, sterile growth media (R2A, ISP2 and V8), sterile TSB, and sterile 0.1 N HCl were also used as negative controls. Each of the UV treated cave bacteria isolate samples had 80 μL pipetted onto each disc, and the discs were then exposed for 30 minutes to UV light (254 nm) in a Labconco Class II biological safety cabinet. Then 80 μL of the non-UV samples and controls were then pipetted onto the discs. All the discs including UV, non-UV and control discs were allowed to dry for 45 minutes.

Large square agar plates were used to carry out the Kirby-Bauer disc diffusion assay. Two plates were used to accommodate all cave bacterial isolates and controls. Two 250 mL flasks were prepared containing 200 mL each of molten Trypticase soy agar which was

autoclaved at 121ºC for 15 minutes. A sterile cotton swab was used to collect *P. aeruginosa* colonies from the TSA plate. During this time two 16x100 mm test tubes had 5 mL of sterile TSB pipetted into them. Then *P. aeruginosa* colonies were collected from a previously cultured TSA plate and deposited into each of the test tubes. Each test tube was brought to a 1.0 McFarland standard using the deposited bacteria. This was achieved by taking a 500 uL sample and using a spectrophotometer at 600 nm (1.0 Mcfarland standard; OD=0.257 at 600 nm). After the molten Trypticase soy agar flasks were autoclaved, the flasks were put into a 55ºC water bath so the molten agar to equilibrate to the same temperature. At this point 2 mL of the prepared *P. aeruginosa* inoculum from one test tube was deposited into the 200 µL of molten TS agar (1% v/v), and then repeated with the other test tube and flask. Each flask was then poured into sterile large square plates and allowed to solidify. The previously prepared discs were then deposited onto the inoculated large square agar plates in alternating rows of 8 and 7 discs (Figure 6). The plates were then incubated at 35ºC for 18 hours, removed and inhibitory zones were measured in millimeters. A sample showing an inhibitory zone was designated as having an inhibitory effect and if the concentration was known, then it was the minimum inhibitory concentration (MIC).

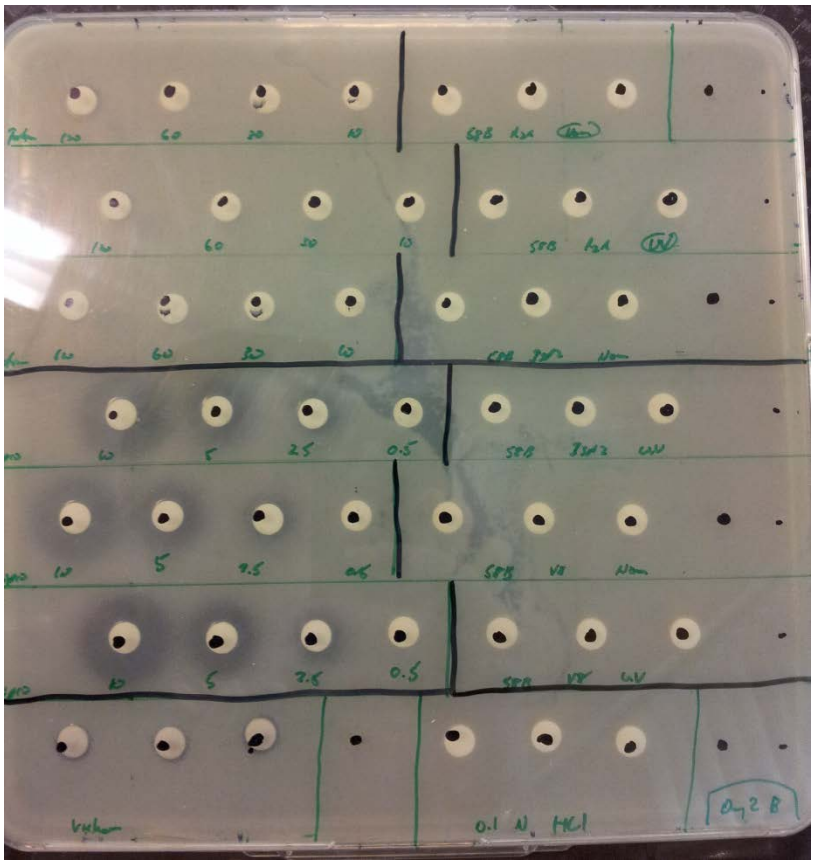

Figure 6: Example of large square agar plates with discs containing cave bacteria isolate samples, and controls used to assess the antimicrobial activity of each sample. The diameters of the dark inhibitory zones that encircle each disc were measured to assess the antimicrobial activity.

Molecular Identification of *Pseudomonas aeruginosa* and Cave Bacterial Isolates by 16S rRNA gene sequencing

In order to prepare for the gene sequencing, fresh agar plates were used to culture the cave bacterial isolates as well as *P. aeruginosa*. The cave bacteria were inoculated onto new HT agar plates, which were then streaked for isolated colonies in order to obtain pure cultures. These plates were incubated at 25^{o}C for 7 days and checked for pure colonies. *P. aeruginosa* (lab strain with unknown strain number) was cultured onto a trypticase soy agar plate using the four way streak method for isolated colonies. The plate was incubated for 18 hours at 35^{o}C, and then inspected after the incubation to ensure that it was a pure culture. The plates

were then sent to Seoul, Korea for 16S rRNA gene sequencing by Macrogen. Sequencing used 518F/800R and 27F/1492R primers and performed with Big Dye terminator cycle sequencing products, which were resolved on Applied Biosystem model 3730XL automated DNA sequencing system at Macrogen. The resulting 16S rRNA sequences were compared with the GenBank database by using BLAST.

RESULTS AND DISCUSSION

Scanning Electron Microscopy for Viewing *Pseudomonas aeruginosa*

The structure of the *P. aeruginosa* biofilm was able to be viewed with the Zeiss Evo LS SEM. This helped establish that the *P. aeruginosa* was indeed in a biofilm formation and that characteristics such as increased antibiotic resistance would be observed in susceptibility tests. The images produced revealed a gradient of single planktonic cells to what appears to be mature biofilms. The initial attached cells were near the top of the peg close to the surface of the culture media. The biofilms started at the bottom of the peg and continued to where the individual cells initially attached. As seen in Figure 7A, starting in the top left of the image, small dots are visible, which would characterize the single planktonic *P. aeruginosa*. Figure 7B shows planktonic cells that appear to have the intermediate shape known as coccobacillus which would represent *P. aeruginosa.* Continuing from the top left to bottom right of Figure 7A, there seems to be aggregation of cells into clusters characteristic of a biofilm, more specifically microcolonies. In this same image, channels are apparent throughout the microcolony and mature biofilm portion. These water filled channels enable enhanced access

of nutrients throughout the biofilm and allow thicker biofilm areas to have enough resources to survive (Costerton et al., 1999; Flemming and Wingender, 2010; Hall-Stoodley et al., 2004). Also, Figure 7C shows a much more rounded structure which appears to be slightly raised from the rest of the biofilm. This mushroom-like structure is usually indicative of a mature portion of the biofilm and has been shown to have high amounts of eDNA (Allesen-Holm et al., 2006). Overall the SEM under partial vacuum produced images with good resolution which could be used to identify components of the biofilm. Similarly, Alhede et al., (2012) used environmental conditions with the SEM to obtain similar images to this study. They used biofilm samples placed in the SEM chamber with minimal preparation and used a partial vacuum.

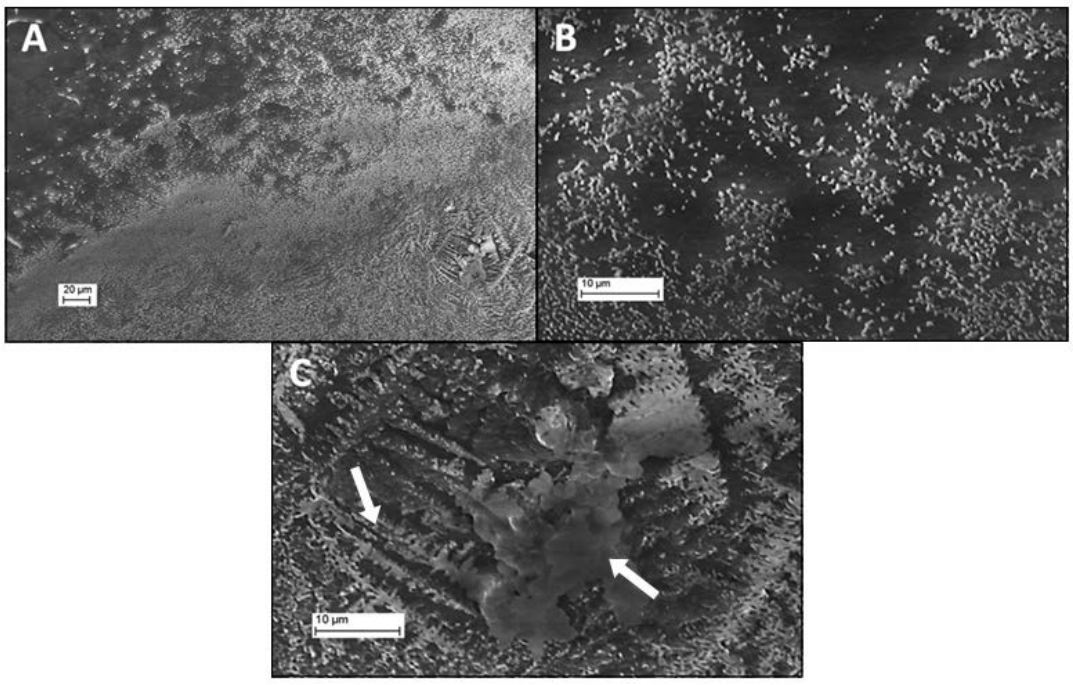

Figure 7: SEM images of *P. aeruginosa* Biofilms viewed with Zeiss Evo LS SEM; A) Image of Biofilm showing planktonic cells to mature biofilms; B) Image of planktonic cells C) Image of mature biofilm with mushroom like colonies(left arrow indicates water channels and right arrow indicates mushroom like colonies)

Antimicrobial Activity of Cave Bacterial Isolates Observed with Spot Plating

All three strains, A1A3, RA003 and 58B showed inhibitory activity against *Pseudomonas aeruginosa* biofilms. Similar to results seen in a previous study (Mason, 2015), these isolates did not show any inhibitory activity until day 8. Previously these strains did not produce metabolites that inhibited biofilms until day 7, and lost the activity by day 11 (Mason, 2015). Furthermore the Kirby-Bauer disc diffusion assay showed similar results with none of the cave isolates exhibiting inhibition of *P. aeruginosa* with sample days 2, 4, 6, 8, and 10. The sample days 2, 4, 6, and 10 had no indication of reduced growth after plating out surviving biofilms so those days did not have the colony numbers enumerated or CFU/mL calculated. Day 8 visually showed reduced or changed colony morphology compared to the growth controls (R2A, ISP2, V8 and TSB). The recovery 96 well plated was used to make dilutions, spot plate the dilutions, and enumerate the colonies to determine the CFU/mL (Figure 8).

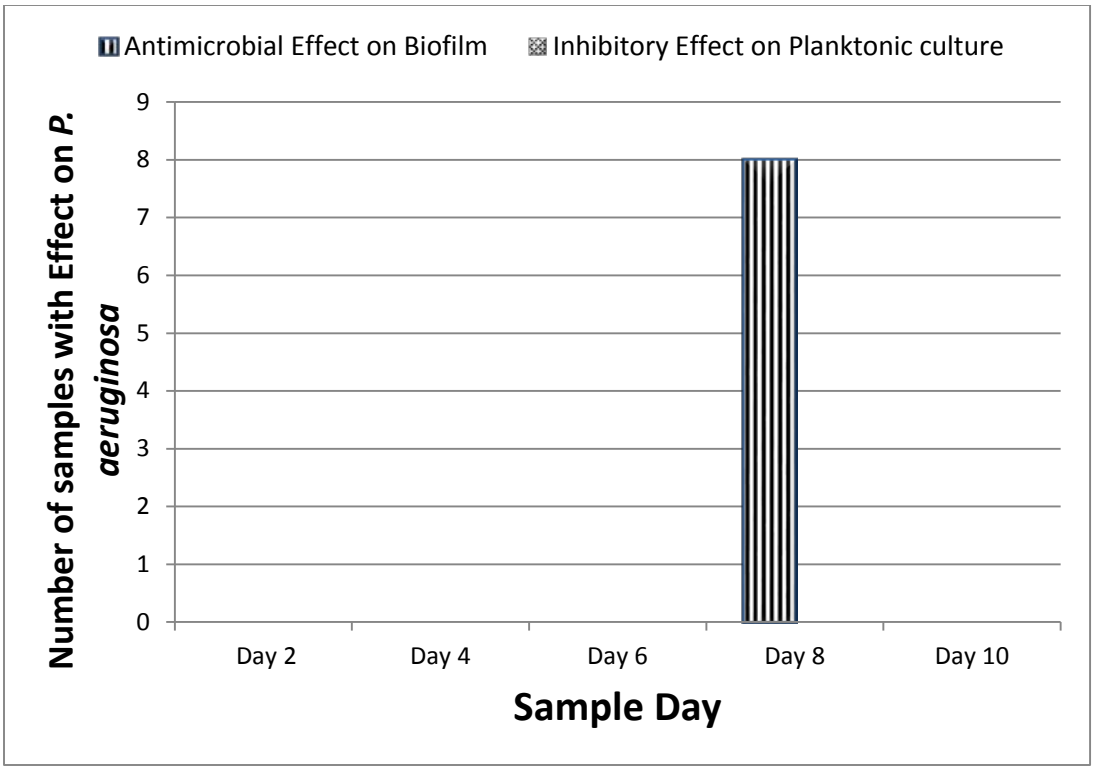

Figure 8: The number of samples produced by the cave bacteria that showed antimicrobial activity against *P. aeruginosa* in a biofilm or planktonic form.

The day 8 samples showed all three strains (A1A3, RA003, and 58B) having an inhibitory effect against the biofilms. This agrees with the preliminary study where all three had inhibitory activity at day 7(Mason, 2015). The colony spots of the cave isolate samples were compared to the TSB control (biofilm only exposed to TSB) colony spots (Figure 9 I). A1A3 cultured in V8, and not exposed to UV (Non-UV) reduced the colony spot in replicate 1. However the replicates 2 and 3 did not show reduced or changed colony morphology compared to the controls (Figure 9 A). The cave isolate RA003 cultured in R2A Non-UV also showed some inhibition in replicate 1 with the colony spot having colonies spread out rather than one solid colony spot. However the replicates 2 and 3 did not show visible inhibition (Figure 9 B). The cave isolate RA003 cultured in ISP2 Non-UV also had a reduced

colony spot, with only a few smaller colonies present. The replicates 2 and 3 had no visible reduction in the colony spot and probably were not affected (Figure 9 C). The cave isolate RA003 cultured in V8 Non-UV showed a smaller colony spot overall indicating some inhibitory effect. The replicates 2 and 3 did not visibly show any inhibition (Figure 9 D). The cave isolate RA003 V8 with UV exposure showed some inhibition with the colony spot of replicate 3 showing a lighter colony colour possibly from reduced cells. The replicates 1 and 2 did not show any inhibition (Figure 9 E). The cave isolate sample 58B R2A Non-UV completely eradicated the biofilm in replicate 1 evident by the no colony spot. The replicates 2 and 3 did not show any visible colony spot reduction (Figure 9 F). The sample 58B V8 Non-UV had replicate 1 having a colony spot with small spread out colonies rather than one large colony spot indicating biofilm inhibition. The replicates 2 and 3 did not visibly show any reduction in the colony spot (Figure 9 G). Lastly the sample 58B V8 UV had replicates 2 and 3 showing no colony spot indicating complete biofilm eradication. However, replicate 1 did not show any inhibition (Figure 9 H) (Appendix B).

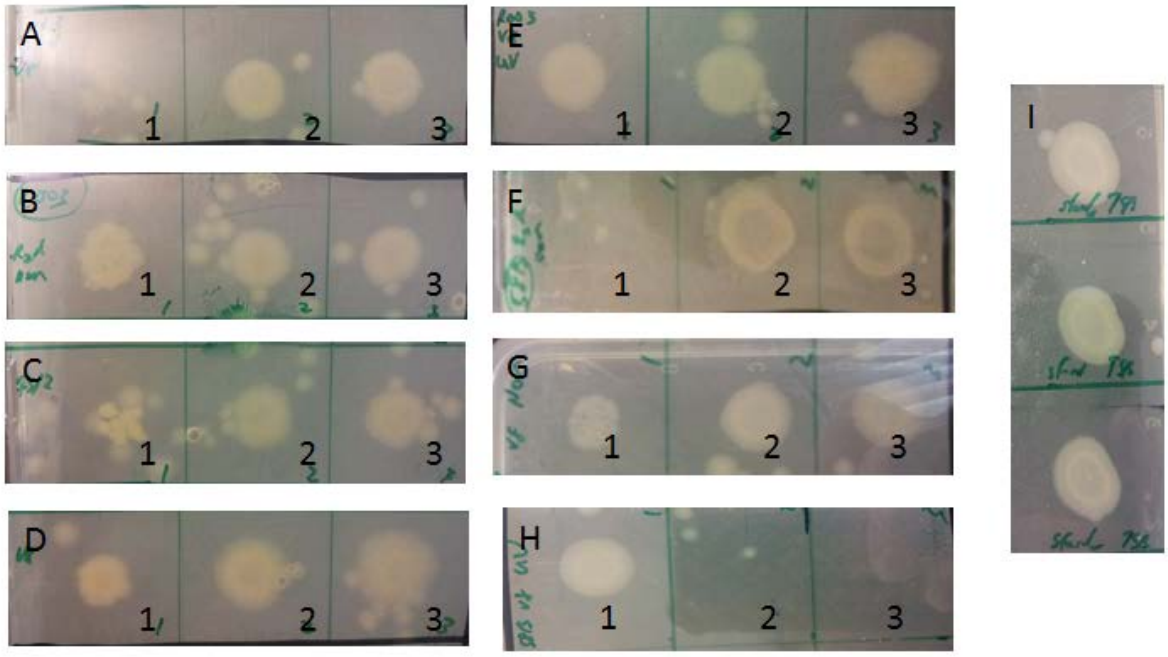

Figure 9: Results of spot plating surviving *P. aeruginosa* biofilms onto TSA plates after exposure to Day 8 samples: A) A1A3 V8 Non-UV B) RA003 R2A Non-UV C) RA003 ISP2 Non-UV D) RA003 V8 Non-UV E) RA003 V8 UV F) 58B R2A Non-UV G) 58B V8 Non-UV H) 58B V8 UV I) Sterile TSB. The numbers 1, 2, and 3 denote replicate number of each sample.

Antimicrobial Activity of Cave Bacterial Isolates Determined with Calculation of CFU/mL

The day 8 samples that visually showed reduction of the biofilm demonstrated by an abnormal or absent colony spot on the TSA plates had the CFU/mL calculated. The calculated CFU/mL for each set of sample replicates agreed with the visual colony spot on the TSA plates. That is, if the colony spot was reduced, than the CFU/mL for that replicate reflected a decreased CFU/mL when compared to the TSB control. The calculated CFU/mL for each growth control (R2A, ISP2 and V8) was compared to the TSB control using a 2 sample T-test to confirm that the media had no effect on the *P. aeruginosa* biofilms. The P-value for each comparison of media to TSB control was greater than 0.05 meaning that there was no significant difference. This means that the R2A, ISP2 and V8 media did not affect the

P. aeruginosa biofilms. The cave bacteria isolate samples were also compared in a similar fashion with the use of a 2 sample T-test. The samples exposed to A1A3 V8 Non-UV, RA003 R2A, ISP2 and V8 Non-UV, 58B R2A Non-UV and RA003 V8 UV all had P-values greater than 0.05 and that the difference in CFU/mL was not significant. The biofilms exposed to 58B V8 Non-UV and 58B V8 UV both showed a significant difference (P-value less than 0.05) between the CFU/mL for these samples compared to the TSB control (Table 1). This would suggest that there was some metabolite in the supernatant samples from the 58B cave isolate cultured in V8 medium and was active with and without UV exposure. The cave isolate 58B cultured in V8 also showed antimicrobial activity against *P. aeruginosa* biofilms (Figure 10).

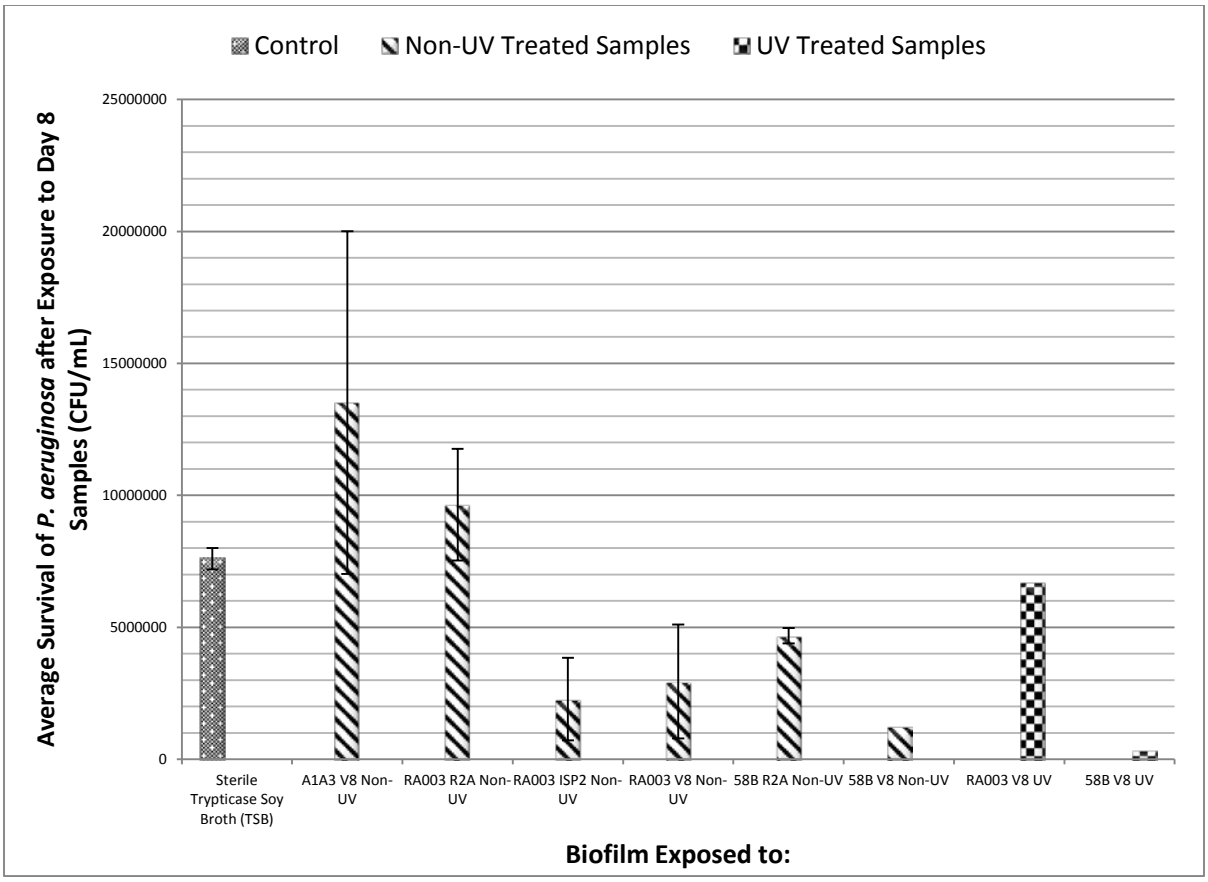

Figure 10: The average survival of *P. aeruginosa* cells (in CFU/mL) compared to the TSB control after exposure to Day 8 samples (n=3). The error bars indicate standard error of the mean.

The samples exposed to A1A3 V8 Non-UV, RA003 R2A, ISP2 and V8 Non-UV, 58B R2A Non-UV and RA003 V8 UV did not show significant reduction of CFU/mL compared to the TSB control. However, at least one replicate of each sample did show a noticeably reduced CFU/mL. A1A3 V8 Non-UV replicate #1 had a CFU/mL of 2.8×10^4 which is noticeably different than the TSB average of 7.6×10^6 CFU/mL. RA003 in R2A, ISP2 and V8 Non-UV also had one replicate with an observable difference; 3.5×10^4, 2.8×10^4, and 4.5×10^4 CFU/mL respectively. As well 58B R2A Non-UV had one replicate of 4.5×10^5 CFU/mL which differs slightly from the TSB control of 7.6×10^6 CFU/mL (Table 1). This

could be due the fact that the three replicates for each sample were actually from separate tubes. The tubes were in the same conditions (temperature, humidity, oxygen, etc.) but small differences inside the tubes could have resulted in different secondary metabolite production, having different antimicrobial efficiency.

Table 1: Table of each sample from Day 8 that had an antimicrobial effect and *P. aeruginosa* biofilms. The surviving cells (CFU/mL) from the biofilms after exposure were calculated from the spot plating the cells. The three replicates were used to find the average CFU/mL and then the average percent survival by taking the sample average and dividing it by the TSB control. The two sample T test (n=3) was used to find if the surviving cell percentage was the same between biofilms exposed to the cave isolate samples and the TSB control.

Sample	Surviving Cells After Exposure (CFU/mL)			Average CFU/mL	Average % survival	P value comparing sample to TSB control	Significantly different than TSB control
	Replicate #1	Replicate #2	Replicate #3				
A1A3 V8 Non-UV	$2.8x10^4$	$2.7x10^7$	$1.2x10^7$	$1.3x10^7$	171.2	0.560	No
RA003 R2A Non-UV	$3.5x10^4$	$6.9x10^6$	$2.2x10^7$	$9.6x10^6$	127.0	0.783	No
RA003 ISP2 Non-UV	$2.8x10^4$	$3.1x10^5$	$6.5x10^6$	$2.3x10^6$	30.0	0.132	No
RA003 V8 Non-UV	$4.5x10^4$	$3.4x10^6$	$5.4x10^6$	$2.9x10^6$	38.8	0.102	No
58B R2A Non-UV	$4.5x10^5$	$7.5x10^6$	$6.1x10^6$	$4.7x10^6$	61.6	0.315	No
58B V8 Non-UV	**$7.8x10^5$**	**$1.2x10^6$**	**$1.8x10^6$**	**$1.3x10^6$**	**16.6**	**0.001**	**Yes**
RA003 V8 UV	$6.8x10^6$	$5.8x10^6$	$7.4x10^6$	$6.7x10^6$	87.7	0.228	No
58B V8 UV	**$1.1x10^6$**	**0**	**0**	**$3.5x10^5$**	**4.6**	**0.001**	**Yes**
R2A sterile	$6.1x10^6$	$6.2x10^6$	$6.8x10^6$	$6.4x10^6$	83.8	0.075	No
ISP2 sterile	$7.8x10^6$	$6.2x10^6$	$5.9x10^6$	$6.6x10^6$	87.3	0.269	No

V8 sterile	$6.4x10^6$	$7.8x10^6$	$7.0x10^6$	$7.1x10^6$	93.0	0.420	No
TSB	$7.1x10^6$	$7.3x10^6$	$8.4x10^6$	$7.6x10^6$	N/A	N/A	N/A

The Antibiotic and Disinfectant Susceptibilities of *P. aeruginosa* in a Biofilm

The controls used for sample days 2, 4, 6, 8, and 10 included 2% Virkon and antibiotics which had different effects in this sample day. The positive control, 2% Virkon, had biocidal properties, and resulted in the complete eradication of the biofilm. After the spot plating the Virkon exposed samples, there was no visible growth on the TSA plate. This would suggest that Virkon completely killed the *P. aeruginosa* biofilm. The biocidal activity of Virkon has previously been studied (Hernndez et al., 2000), and found that it is effective after 5 minutes of contact time with bacteria. Hernndez et al., (2000) found that 5 minutes of contact to *P. aeruginosa* had complete biocidal activity, and therefore 0 CFU/mL. Furthermore this study showed that *P. aeruginosa* biofilms exposed to Virkon for 18 hours also resulted in 0 CFU/mL. Tetracycline HCl (120, 60, 30, and 10 μg/mL) and ciprofloxacin (10, 5, 2.5 and 0.5 μg/mL) were both used but showed different effectiveness.

The antibiotic tetracycline HCl at a 120, 60, 30, and 10 μg/mL had no inhibitory effect against the *P. aeruginosa* biofilms. The colony spot of the biofilm exposed to tetracycline even at a concentration as high as 120 μg/mL did not show any sign of inhibition. Tetracycline is a broad spectrum antibiotic with activity against Gram positive and Gram negative bacteria. It works by inhibiting protein synthesis by preventing the attachment aminoacyl-tRNA to the ribosomal acceptor (A) site (Chopra and Roberts, 2001). However *P. aeruginosa* is intrinsically resistant to tetracycline due the MexAB/MexXY efflux systems.

The efflux pumps remove the molecule from the cell before it can reach toxic concentrations (Morita et al., 2001) therefore tetracycline is generally not used against *P. aeruginosa*.

The antibiotic ciprofloxacin (dissolved in 0.1 N HCl) at 10, 5, 2.5 and 0.5 μg/mL was used as a positive control on days 2, 4, 6, and 8. This antibiotic showed no colony spots on the TSA plates indicating biocidal activity. However, the control with only 0.1 N HCl also had no colony spot indicating complete biocidal activity. This would suggest that it was not the ciprofloxacin alone that was able to eradicate the *P. aeruginosa* biofilms. Most likely the 0.1 N HCl did not having enough nutrients to sustain the cells. The Kirby-Bauer disc diffusion assay (discussed later) showed that 0.1 N HCl had no inhibitory effect on *P. aeruginosa* in a planktonic state. However with the day 10 samples, ciprofloxacin HCl was used (dissolved in water) as a positive control at 10, 5, 2.5 and 0.5 μg/mL. When ciprofloxacin HCl was used against *P. aeruginosa* biofilms, it had biocidal effects. Since ciprofloxacin is dissolved in water, it was most likely the antibiotic that had biofilm eradication effect. At concentrations of 10 and 5 μg/mL it had complete biocidal activity and eradicated the biofilm resulting in 0 CFU/mL. The antibiotic is a fluoroquinolone and targets DNA gyrase which then inhibits cell division (Poole, 2011). The 5 μg/mL concentration represents the MBEC of ciprofloxacin HCl on *P. aeruginosa*. This MBEC agrees with other values found which put the MBEC of *P. aeruginosa* (ATCC 27853) at 4 μg/mL (Ceri et al., 1999) but a more recent study put it in the range of 0.25-8 μg/mL (same strain) (Dosler and Karaaslan, 2014).

Table 2: The surviving cells (CFU/mL) of the *P. aeruginosa* biofilms after exposure to Ciprofloxacin HCl (day 10 exposures).

Ciprofloxacin HCl (µg/mL)	Replicate #1	Replicate #2	Replicate #3	Average CFU/mL
10	0	0	0	0
5	0	0	0	0
2.5	$5.6x10^5$	$8.0x10^5$	$7.2x10^5$	$6.9x10^5$
0.5	$4.7x10^5$	$5.6x10^5$	$4.3x10^5$	$4.9x10^5$

Antimicrobial Activity of cave Bacterial Isolates on Planktonic *P. aeruginosa*

The day 2, 4, 6, 8 and 10 samples were also used in a KB disc diffusion assay but none of the strains in any sample demonstrated any inhibition of *P. aeruginosa* on agar plates. Oddly even day 8 samples which exhibited antimicrobial effects against the biofilms, did not have an effect on planktonic *P. aeruginosa*. The day 8 samples were used in the KB disc diffusion assay 5 days after they were used in the Biofilm assay. When the samples were initially used, they were stored at -20ºC, but after they were stored at 4ºC, so it is possible that the higher temperature resulted in chemical changes to the molecules in the samples, rendering them ineffective against *P. aeruginosa*. However, an alternative idea is that the cave isolate supernatant inhibited the biofilm rather than killing the cells. Since the supernatant samples did not inhibit the planktonic cells, but did reduce the biofilms, it is possible that the cave isolate supernatants actually were able to disrupt the biofilms without killing the cells within.

The Antibiotic and Disinfectant Susceptibilities of *P. aeruginosa* in a Planktonic Form

The same positive controls of 2% Virkon, tetracycline HCl, and ciprofloxacin (HCl) were used with the same concentrations as the biofilm assays. The 2% Virkon in day 2, 4, 6, 8, and 10 showed an inhibitory zone (too small to measure) around the discs. The diameter may have been small because of the chemical composition of Virkon. Virkon is composed of peroxygen compounds including Potassium peroxymonosulfate, which is a negatively charged particle, making it difficult to diffuse through the agar, which is also negatively charged. This idea is again consistent with that of Hernndez et al., (2000), which found that Virkon had bactericidal effects on *P. aeruginosa*.

The antibiotic tetracycline HCl also showed inhibitory activity when used in the KB disc diffusion assay. For days 2, 4, 6, 8, and 10, tetracycline HCl at 120 μg/mL showed an inhibitory zone. Days 2, 4, and 8 showed measureable inhibitory zones that ranged from 10 to 14 mm. Also Days 4 and 8 showed inhibitory zones at 60 μg/mL, but were too small to measure. So the 60 μg/mL could be thought of as the MIC of tetracycline HCl on *P. aeruginosa*. This is within the range of other MIC's found for *P. aeruginosa* (various lab strains such as PA01) which were 0.5-64 μg/mL (Li et al., 1994; Morita et al., 2001).

The antibiotic ciprofloxacin (dissolved in 0.1 N HCl) was used for sample days 2, 4, 6, and 8 and ciprofloxacin HCl was used for sample day 10. Both showed very similar inhibitory activity when used in the KB disc diffusion assay. A control with just 0.1 N HCl was used for days 2, 4, 6, and 8 which showed that it had no inhibitory activity (a different result than the Biofilm assay mentioned earlier). For sample days 2, 4, 6, 8, and 10 the

ciprofloxacin/ciprofloxacin HCl showed inhibitory zone ranges of 26-19 mm and 18-10 mm for concentrations of 10 and 5 μg/mL respectively. Ciprofloxacin/ciprofloxacin HCl at 2.5 μg/mL showed an inhibitory zone of 12-10 mm for Day 2 then inhibitory zones too small to measure for Day 4, 6, 8, and 10. The concentration of 0.5 μg/mL had no inhibitory zone, indicating too low of a concentration to have an inhibitory effect on *P. aeruginosa*. This would indicate that 2.5 μg/mL is the MIC which is slightly higher than some other studies have found. Some studies put the MIC in a range of 0.1-0.5 μg/mL for various strains of *P. aeruginosa* including ATCC 27853 and PA01 (Ceri et al., 1999; Li et al., 1994; Morita et al., 2001), but a more recent study showed an MIC range of 0.25-2 μg/mL (Dosler and Karaaslan, 2014), which is a value much closer to what this study found.

Antimicrobial Activity as Compared to Previous Studies with Same Isolates

The results of this study correspond to earlier studies conducted with the cave isolates A1A3, RA003 and 58B. In a preliminary study, these cave isolates showed the ability to inhibit *P. aeruginosa* biofilms resulting in a reduced CFU/mL (Mason, 2015). The preliminary study found 58B V8 Non-UV as well as RA003 and A1A3 R2A Non-UV had inhibitory effects on biofilms. That study (Mason, 2015) and this one both show that biofilm inhibitory molecules are produced by the strains starting on day 7 (result of previous study) and day 8 (this study) but lose the activity by day 10 as shown in this study. The current study found that A1A3, RA003 and 58B could produce compounds that could inhibit *P. aeruginosa* biofilms. The cave isolate RA003 could produce antimicrobial compounds when cultured in R2A, ISP2 and V8 as well as being effective after the supernatant was exposed to UV light. This would suggest that RA003 is versatile organism that can grow in a variety of

culture media. In the preliminary study, RA003 was found to have inhibitory activity only in R2A. The cave isolate 58B was able to produce antimicrobial compounds when cultured in R2A and V8 as well. Previously in the preliminary study, 58B was only able to have inhibitory effects when cultured in V8. The cave isolate A1A3 was only able to produce antimicrobial compounds when cultured in V8. However in the preliminary study, it produced antimicrobial compounds in R2A (Mason, 2015).

These cave isolates also have similar antimicrobial activities compared to previous studies conducted with these strains. The cave isolate A1A3 had antimicrobial effects against the Gram-negative bacteria *Klebsiella pneumoniae*, *Escherichia coli* and *Acinetobacter baumannii* having inhibitory zones of 11-13 mm when cultured in V8 (Sadoway, 2011) similar to this study. Also RA003 has shown activity against *Micrococcus luteus* and MDR-MRSA when cultured in R2A, HT and ISP2, producing zones of inhibition 11-15mm (Alnahdi, 2014). This shows again that the cave isolate RA003 is able to produce antimicrobial compounds in a variety of media. Rule, (2012) found the cave isolate 58B had antimicrobial effects against *Mycobacterium smegmatis* and *A. baumannii* when cultured in HT. Sadoway, (2011) found that cave isolate 58B had antimicrobial effects on *E. coli* but only in HT and ISP2, not when cultured in V8 medium. Furthermore Alnahdi, 2014 found that the cave bacteria isolates cultured in R2A medium had high antimicrobial activities. Her study showed the 58B cave isolate cultured in R2A had antimicrobial effects against *Micrococcus luteus, M. smegmatis*, and *Candida albicans*. In addition, the RA003 cave isolate had antimicrobial activity against *M. luteus* when cultured in R2A medium. Alnahdi, (2014) identified R2A broth to be the optimal fermentation broth because the zones of inhibition for 58B and RA003 were larger when grown in R2A as compared to HT broth.

This study showed R2A was able to support isolates 58B and RA003 to make antimicrobial compounds, but it did not seem to be the most effective culture medium. The V8 culture medium was able to sustain A1A3, RA003 and 58B, which caused all three strains to produce compounds that could inhibit *P. aeruginosa* biofilms. The most interesting finding was that 58B cultured in the V8 medium produced significant reduction of the biofilms with and without UV treatment. In fact, the UV treated 58B V8 supernatant yielded a compound that in two replicates resulted in complete biofilm eradication, an impressive result considering an impure sample.

Furthermore this study found that only one of the cave isolates showed antimicrobial activity after exposure to UV light for 30 minutes; however, it was the most effective sample. Sadoway, (2011) found that the cave isolate 58B only had an antimicrobial effect against *E. coli* when not exposed to UV. Also Rule, (2012) found that the same cave isolate again only had activity against *M. smegmatis* and *A. baumannii* when not exposed to UV light. In this study, it is possible that the UV light actually increased the antimicrobial compound's antimicrobial effect by causing a conformation change. One study showed that the antibiotics oxytetracycline, doxycycline and ciprofloxacin had increased inhibition against *Vibrio fisheri* when exposed to 3816 mJ/cm^2 of UV fluence (energy rate) (Yuan et al., 2011).

Identities of Cave Bacterial Isolates and *P. aeruginosa*

The clinically useful antibiotics used to treat *P. aeruginosa* are generally carbapenems, fluoroquinolones, and aminoglycosides (Morita et al., 2014). Since these cave bacterial isolates produced antimicrobial compounds that inhibited *P. aeruginosa*, it is possible that the compounds may resemble one of the previous antibiotics mentioned. In fact,

carbapenems and aminoglycosides have been known to be produced by *Streptomyces* species (Poole, 2011). The cave isolates A1A3 and 58B were shown to be Gram positive filamentous bacteria, which is consistent with *Streptomyces* species identification. Furthermore the 16S rRNA sequencing also showed that both A1A3 and 58B were *Streptomyces* species with 99% shared similarity and 100% query coverage (Table 3). However due to the similarity and query coverage, a species could not be discerned for either isolate. The cave isolate RA003 was a Gram positive rod shaped bacterium and the 16S rRNA sequencing agreed with this. The sequencing showed that the cave isolate RA003 was in the *Bacillus* genus but again due the similarity and query coverage a species could not be discerned (Table 3). The gene sequencing also showed that the pathogenic bacterium was *P. aeruginosa* but a strain could not be concluded based on the similarity and query coverage (Table 3). Members of the *Bacillus* genus has previously been able to produce bacteriocins (antimicrobial peptides) against a wide variety of bacteria. Some examples the bacterium's inhibitory effects are: *Listeria monocytogenes* ATCC 19111, *Pseudomonas fluorescens* ATCC 11251, *Staphylococcus aureus* ATCC 25923 and *E. coli* (Abdel-Mohsein et al., 2011; He et al., 2006; Kayalvizhi and Gunasekaran, 2008).

Table 3: Identification of the organisms used according to 16S rRNA gene sequencing

Organism Analyzed	Possible Identity	Percent Similarity	Query Coverage
A1A3	*Streptomyces fulvissimus* strain DSM 40593	99	100
	Streptomyces microflavus strain PM86A	99	100
58B	*Streptomyces fulvissimus* strain DSM 40593	99	100
	Streptomyces microflavus strain PM86A	99	100

RA003	*Bacillus pumilus* strain Jo2	99	100
	Bacillus altitudinis strain KUDC1731	99	100
Pseudomonas	*Pseudomonas aeruginosa* strain SBTPe-001	99	100
aeruginosa	*Pseudomonas aeruginosa* strain SV1	99	100
	Pseudomonas aeruginosa strain VSS6	99	100

Microbial natural products are still a major source of new drugs for antibiotic production, but must be discovered first. Microorganisms from a wide variety of environments have been shown to be able to combat pathogenic bacteria and in some cases the biofilms they form. The biofilms are quite often a major cause of the pathogenicity of the bacterium in question. The bacterial strains used in this study were isolated from caves found in British Columbia, which further solidifies that natural habitats can produce many useful bacteria. Recently, a study used bacteria from soil, cave, and rivers to produce cell free extracts. The extracts were then used against *Staphylococcus aureus* biofilms in a 96 well microtitre plate, similar to this study. The study found that 55/126 extracts significantly inhibited the biofilms due a variety of chemical compounds found in the extracts. Also 40% of the extracts were found to contain DNase, which could break down extracellular DNA, a major structural component of some biofilms (Farmer et al., 2014). In another study, researchers collected bacterial samples from Magura Cave in Bulgaria. The samples were very diverse with respect to bacterial species, and all produced varying compounds such as proteases, xanthan lyase and β-glycosidase. Again the researchers found that 75% of the collected samples showed antimicrobial activity against *Bacillus subtilis* ATCC 6633 and *Pseudomonas aeruginosa* NBIMCC 1390 in a biofilm (Lazarkevich et al., 2013). Some

marine bacterial isolates have been studied as well for their biofilm effects. In one study bacterial strains from sediment in the Palk Bay region were isolated, then used to produce cell free extracts. These extracts did not show biofilm biocidal effects, but could inhibit the biofilms from forming. The marine isolates *Bacillus indicus* MTCC 5559 and *Bacillus pumilus* MTCC 5560 showed mature biofilms of *P. aeruginosa* being inhibited by 70-74% (Nithya et al., 2010).

CONCLUSIONS AND FUTURE WORK

Cave bacteria, including actinomycetes, continue to be important in the discovery of new bioactive compounds. The cave bacteria isolates A1A3, RA003, and 58B used in this project continued to have inhibitory activity against another pathogenic bacteria *Pseudomonas aeruginosa*. Of the 90 total supernatant samples collected from culturing for 2, 4, 6, 8 and 10 days (3 strains, 3 different media, 2 treatments, and 5 different days) only 8 of them showed inhibitory activity (approximately 9%) . These samples were all from the Day 8 collection, but only 2 of the samples showed significant inhibition of the *P. aeruginosa* biofilms. Since the samples had no effect on the planktonic cells, it is reasonable to think that there was a biofilm dispersal effect rather than a bactericidal effect. The fermentation broths of R2A and V8 seemed to be the most effective choices to produce secondary metabolites from cave bacteria. The V8 medium did yield antimicrobial secondary metabolites from all three strains used. The exposure of UV light did appear to have an activating effect on the samples RA003 and 58B both cultured in V8 and 58B turned out to be the more effective, having complete biofilm eradication properties. It is promising that these cave bacterial isolates produced secondary metabolites that would inhibit biofilms at all. Cells living in a

biofilm are more resistant to antimicrobial compounds, so it is impressive that the cave isolates could have inhibitory effects without any purification or concentrating. The next step would be to take samples from Day 7 to Day 9 in order to pinpoint where the most effective secondary metabolite it produced. The culture conditions such as temperature, pH, oxygen etc. could also be studied to optimize the antimicrobials produced. As well it would be important to purify and identify what specific molecules are present in the cave isolate samples in order to decipher the mechanism of action.

It is obvious now that antibiotic susceptibilities of free-living bacteria are greatly reduced as compared to biofilm susceptibilities. The MIC of *P. aeruginosa* to the antibiotic ciprofloxacin HCl is lower (half according to this study) than what is need for the MBEC. Using the MBEC 96 well microtitre plate to study susceptibilities of biofilms to antimicrobials is essential. This high throughput method allows for more realistic conditions, giving more accurate susceptibilities of the organisms in their natural state. Plate counts were used in this study to evaluate the biofilm eradication activity of the cave isolates. This method is useful because it can distinguish between live and dead cells to give accurate cell concentration measurements. However, it is incredibly time intensive, and sometimes prone to contamination, which meant doing the trial over again. Using a plate reader would be much faster and more efficient than using plate counts. It would be best to use the plate reader for the initial biocidal activity, and then confirm the result with the plate counts. This would make the process more efficient while still retaining accurate results of whether the cells are alive or dead. As well this study used a laboratory strain of *P. aeruginosa* to evaluate the antimicrobial activity of the cave isolate samples. The next step may be to use

multi-drug resistant strains of *P. aeruginosa* from hospitals and determine if the cave bacterial isolates are still effective against those strains.

Overall, the three cave bacteria isolates: A1A3, RA003, and 58B were able to have an inhibitory effect against *P.* aeruginosa biofilms. The Kirby-Bauer disc diffusion assay determined that the cave bacterial isolates did not have an effect on planktonic cells. The result of these two experiments would suggest that the cave isolates contain biofilm disrupting compounds. The scanning electron microscopy found that the bacteria did grow in a biofilm formation on the pegs of the MBEC device. The biofilms proved to be more resistant to antibiotics as compared to the planktonic cells. Lastly, the 16s rRNA sequencing identified the cave isolates A1A3, RA003 and 58B as the genera *Streptomyces, Streptomyces* and *Bacillus* respectively.

LITERATURE CITED

Abdel-Mohsein, H.S., Sasaki, T., Tada, C., and Nakai, Y. (2011). Characterization and partial purification of a bacteriocin-like substance produced by thermophilic *Bacillus licheniformis* H1 isolated from cow manure compost. Anim. Sci. J. *82*, 340–351.

Alhede, M., Qvortrup, K., Liebrechts, R., Høiby, N., Givskov, M., and Bjarnsholt, T. (2012). Combination of microscopic techniques reveals a comprehensive visual impression of biofilm structure and composition. FEMS Immunol. Med. Microbiol. *65*, 335–342.

Allesen-Holm, M., Barken, K.B., Yang, L., Klausen, M., Webb, J.S., Kjelleberg, S., Molin, S., Givskov, M., and Tolker-Nielsen, T. (2006). A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. Mol. Microbiol. *59*, 1114–1128.

Alnahdi, R. (2014). Potential of cave bacteria in drug discovery: investigation of *Sphingopyxis terrae* and other bacteria from a BC cave. Masters Sci. Thesis TRU Kamloops BC Can.

Baquero, F. (2001). Low-level antibacterial resistance: a gateway to clinical resistance. Drug Resist. Updat. Rev. Comment. Antimicrob. Anticancer Chemother. *4*, 93–105.

Bauer, A.W., Kirby, W.M., Sherris, J.C., and Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. *45*, 493–496.

Bérdy, J. (2005). Bioactive Microbial Metabolites. J. Antibiot. (Tokyo) *58*, 1–26.

Ceri, H., Olson, M.E., Stremick, C., Read, R.R., Morck, D., and Buret, A. (1999). The Calgary Biofilm Device: New Technology for Rapid Determination of Antibiotic Susceptibilities of Bacterial Biofilms. J. Clin. Microbiol. *37*, 1771–1776.

Cheeptham, N., and Towers, G.H.N. (2002). Light-mediated activities of some Thai medicinal plant teas. Fitoterapia *73*, 651–662.

Cheeptham, N. (Ann), Sadoway, T., Rule, D., Watson, K., Moote, P., Soliman, L., Azad, N., Donkor, K., and Horne, D. (2013). Cure from the cave: volcanic cave actinomycetes and their potential in drug discovery. Int. J. Speleol. *42*.

Chopra, I., and Roberts, M. (2001). Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance. Microbiol. Mol. Biol. Rev. *65*, 232–260.

Coates, A.R.M., and Hu, Y. (2007). Novel approaches to developing new antibiotics for bacterial infections. Br. J. Pharmacol. *152*, 1147–1154.

Costerton, J.W., Stewart, P.S., and Greenberg, E.P. (1999). Bacterial Biofilms: A Common Cause of Persistent Infections. Science *284*, 1318–1322.

Dosler, S., and Karaaslan, E. (2014). Inhibition and destruction of *Pseudomonas aeruginosa* biofilms by antibiotics and antimicrobial peptides. Peptides *62*, 32–37.

Farmer, J. t., Shimkevitch, A. v., Reilly, P. s., Mlynek, K. d., Jensen, K. s., Callahan, M. t., Bushaw-Newton, K. l., and Kaplan, J. b. (2014). Environmental bacteria produce abundant and diverse antibiofilm compounds. J. Appl. Microbiol. *117*, 1663–1673.

Fernández, L., Breidenstein, E.B.M., and Hancock, R.E.W. (2011). Creeping baselines and adaptive resistance to antibiotics. Drug Resist. Updat. *14*, 1–21.

Flemming, H.-C., and Wingender, J. (2010). The biofilm matrix. Nat. Rev. Microbiol. *8*, 623–633.

Gaddy, J.A., and Actis, L.A. (2009). Regulation of *Acinetobacter baumannii* biofilm formation. Future Microbiol. *4*, 273–278.

Gellatly, S.L., and Hancock, R.E.W. (2013). *Pseudomonas aeruginosa*: new insights into pathogenesis and host defenses. Pathog. Dis. *67*, 159–173.

Genilloud, O., González, I., Salazar, O., Martín, J., Tormo, J.R., and Vicente, F. (2011). Current approaches to exploit actinomycetes as a source of novel natural products. J. Ind. Microbiol. Biotechnol. *38*, 375–389.

Hall, B.G., and Barlow, M. (2004). Evolution of the serine beta-lactamases: past, present and future. Drug Resist. Updat. Rev. Comment. Antimicrob. Anticancer Chemother. *7*, 111–123.

Hall-Stoodley, L., Costerton, J.W., and Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. Nat. Rev. Microbiol. *2*, 95–108.

Hauser, A.R. (2009). The Type III Secretion System of *Pseudomonas aeruginosa*: Infection by Injection. Nat. Rev. Microbiol. *7*, 654–665.

He, L., Chen, W., and Liu, Y. (2006). Production and partial characterization of bacteriocin-like pepitdes by *Bacillus licheniformis* ZJU12. Microbiol. Res. *161*, 321–326.

Hernndez, A., Martró, E., Matas, L., Martín, M., and Ausina, V. (2000). Assessment of in-vitro efficacy of 1% Virkon® against bacteria, fungi, viruses and spores by means of AFNOR guidelines. J. Hosp. Infect. *46*, 203–209.

Kayalvizhi, N., and Gunasekaran, P. (2008). Production and characterization of a low-molecular-weight bacteriocin from *Bacillus licheniformis* MKU3. Lett. Appl. Microbiol. *47*, 600–607.

Kim, I., and Tanaka, H. (2009). Photodegradation characteristics of PPCPs in water with UV treatment. Environ. Int. *35*, 793–802.

Kipnis, E., Sawa, T., and Wiener-Kronish, J. (2006). Targeting mechanisms of *Pseudomonas aeruginosa* pathogenesis. Médecine Mal. Infect. *36*, 78–91.

Lam, K.S. (2006). Discovery of novel metabolites from marine actinomycetes. Curr. Opin. Microbiol. *9*, 245–251.

Lazarkevich, I., Tomova, A., Kambourova, M., and Vasileva-Tonkova, E. (2013). Diversity and biosynthetic potential of culturable aerobic heterotrophic bacteria isolated from Magura Cave, Bulgaria. Int. J. Speleol. *42*.

Li, X.Z., Livermore, D.M., and Nikaido, H. (1994). Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa:* resistance to tetracycline, chloramphenicol, and norfloxacin. Antimicrob. Agents Chemother. *38*, 1732–1741.

Mah, T.-F.C., and O'Toole, G.A. (2001). Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. *9*, 34–39.

Mason, C. (2015). Screening of cave bacteria for antimicrobial activity against *Pseudomonas aeruginosa* biofilms: UREAP Report.

Morita, Y., Kimura, N., Mima, T., Mizushima, T., and Tsuchiya, T. (2001). Roles of MexXY- and MexAB-multidrug efflux pumps in intrinsic multidrug resistance of *Pseudomonas aeruginosa* PAO1. J. Gen. Appl. Microbiol. *47*, 27–32.

Morita, Y., Tomida, J., and Kawamura, Y. (2014). Responses of *Pseudomonas aeruginosa* to antimicrobials. Antimicrob. Resist. Chemother. *4*, 422.

Nithya, C., Begum, M.F., and Pandian, S.K. (2010). Marine bacterial isolates inhibit biofilm formation and disrupt mature biofilms of *Pseudomonas aeruginosa* PAO1. Appl. Microbiol. Biotechnol. *88*, 341–358.

Poole, K. (2011). *Pseudomonas aeruginosa*: Resistance to the Max. Front. Microbiol. *2*.

Rule, D. (2012). The effects of UV light on the antimicrobial activities of cave actinomycete metabolites and the examination of these bacteria with "environmental" scanning electron microscopy ("ESEM"). Bachelors Sci. Thesis TRU Kamloops BC Can.

Sadoway, T. (2011). The susceptibility of multidrug resistant, Gram-negative pathogens to antimicrobial compounds produced by cave actinomycetes. Bachelors Sci. Thesis TRU Kamloops BC Can.

Schurek, K.N., Breidenstein, E.B.M., and Hancock, R.E.W. (2012). *Pseudomonas aeruginosa*: A Persistent Pathogen in Cystic Fibrosis and Hospital-Associated Infections. In Antibiotic Discovery and Development, T.J. Dougherty, and M.J. Pucci, eds. (Springer US), pp. 679–715.

Singh, R., Ray, P., Das, A., and Sharma, M. (2010). Penetration of antibiotics through *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. J. Antimicrob. Chemother. *65*, 1955–1958.

Upadhyay, R.K., Dwivedi, P., and Ahmad, S. (2010). Antimicrobial activity of photo-activated cow urine against certain pathogenic bacterial strains. Afr. J. Biotechnol. *9*, 518–522.

Wilkins, T.D., Holdeman, L.V., Abramson, I.J., and Moore, W.E.C. (1972). Standardized Single-Disc Method for Antibiotic Susceptibility Testing of Anaerobic Bacteria. Antimicrob. Agents Chemother. *1*, 451–459.

Yang, L., Liu, Y., Wu, H., Song, Z., Høiby, N., Molin, S., and Givskov, M. (2012). Combating biofilms. FEMS Immunol. Med. Microbiol. *65*, 146–157.

Yuan, F., Hu, C., Hu, X., Wei, D., Chen, Y., and Qu, J. (2011). Photodegradation and toxicity changes of antibiotics in UV and UV/H2O2 process. J. Hazard. Mater. *185*, 1256–1263.

(2014). Standard Test Method for Testing Disinfectant Efficiacy against Pseudomonas aeruginosa Biofilm using the MBEC Assay.

APPENDIX A-MEDIA

Hickey-Tresner Media	
Yeast Extract	1.0 g
Beef Extract	1.0 g
N-Z Amine A	2.0 g
Dextrin	10.0 g
dH_2O	to 1 L
pH	7.3
***Media heated to dissolve Dextrin**	

V8 Juice Media	
V8 supernatant	200.0 mL
$CaCO_3$	3.0 g
dH_2O	800.0 mL
pH	6.0
***PC Blue Ribbon Low Sodium V8 juice centrifuged at 10,000 rpm for 10 minutes to collect supernatant**	

International *Streptomyces* Project # 2 (Yeast-Malt Extract)	
Yeast Extract	4.0 g
Glucose	4.0 g
Malt Extract	10.0 g
dH_2O	to 1 L
pH	7.3

R2A Media	
R2A pre-made broth media	3.15 g
dH_2O	to 1 L
pH	7.0

D/E Neutralizing Broth Media	
Pre-made D/E Neutralizing broth media	34.0 g
dH_2O	to 1 L
pH	7.6

Trypticase Soy Broth Media	
BBL pre-made Trypticase soy broth media	30.0 g
dH_2O	to 1 L
pH	7.3

APPENDIX B-RAW DATA

Day 2 Biofilm recovery growth check (+=Growth, -= No growth, RED= reduced growth)

Non	A1A3 R2A NON	A1A3 ISP2 NON	A1A3 V8 NON	R003 R2A NON	R003 ISP2 NON	R003 V8 NON	58B R2A NON	58B ISP2 NON	58B V8NON
#1	+	+	+	+	+	+	+	+	+
#2	+	+	+	+	+	+	+	+	+
#3	+	+	+	+	+	+	+	+	+
UV	A1A3 R2A	A1A3 ISP2	A1A3 V8	R003 R2A	R003 ISP2	R003 V8	58B R2A	58B ISP2	58B V8
#1	+	+	+	+	+	+	+	+	+
#2	+	+	+	+	+	+	+	+	+
#3	+	+	+	+	+	+	+	+	+
Antibiotic	**Tetra HCl 120 ug/mL**	60	30	10		**Cipro** 10 ug/mL	5	2.5	0.5
#1	+	+	+	+		-	-	-	-
#2	+	+	+	+		-	-	-	-
#3	+	+	+	+		-	-	-	-
	R2A sterile	**ISP2 sterile control**	**V8 sterile control**	**Sterile TSB**	**2% Virkon**	**0.1 N HCl**			
1#	**-**	**-**	**-**	**+**	**-**	**-**			
#2	**-**	**-**	**-**	**+**	**-**	**-**			
#3	**-**	**-**	**-**	**+**	**-**	**-**			

Day 4 Biofilm recovery growth check (+=Growth, -= No growth, RED= reduced growth)

Non	A1A3	A1A3	A1A3 V8	R003	R003	R003 V8	58B R2A	58B ISP2	58B

	R2A NON	ISP2 NON	NON	R2A NON	ISP2 NON	NON	NON	NON	V8NON
#1	+	+	+	+	+	+	+	+	+
#2	+	+	+	+	+	+	+	+	+
#3	+	+	+	+	+	+	+	+	+
UV	A1A3 R2A	A1A3 ISP2	A1A3 V8	R003 R2A	R003 ISP2	R003 V8	58B R2A	58B ISP2	58B V8
#1	+	+	+	+	+	+	+	+	+
#2	+	+	+	+	+	+	+	+	+
#3	+	+	+	+	+	+	+	+	+
Antibiotic	**Tetra HCl 120 ug/mL**	60	30	10		**Cipro** 10 ug/mL	5	2.5	0.5
#1	+	+	+	+		-	-	-	-
#2	+	+	+	+		-	-	-	-
#3	+	+	+	+		-	-	-	-
	R2A sterile	**ISP2 sterile control**	**V8 sterile control**	**Sterile TSB**	**2% Virkon**	**0.1 N HCl**			
1#	**-**	**-**	**-**	**+**	**-**	**-**			
#2	**-**	**-**	**-**	**+**	**-**	**-**			
#3	**-**	**-**	**-**	**+**	**-**	**-**			

Day 6 Biofilm recovery growth check (+=Growth, -= No growth, RED= reduced growth)

Non	A1A3 R2A NON	A1A3 ISP2 NON	A1A3 V8 NON	R003 R2A NON	R003 ISP2 NON	R003 V8 NON	58B R2A NON	58B ISP2 NON	58B V8NON
#1	+	+	+	+	+	+	+	+	+
#2	+	+	+	+	+	+	+	+	+
#3	+	+	+	+	+	+	+	+	+

UV	A1A3 R2A	A1A3 ISP2	A1A3 V8	R003 R2A	R003 ISP2	R003 V8	58B R2A	58B ISP2	58B V8
#1	+	+	+	+	+	+	+	+	+
#2	+	+	+	+	+	+	+	+	+
#3	+	+	+	+	+	+	+	+	+
Antibiotic	**Tetra HCl 120 ug/mL**	60	30	10		**Cipro** 10 ug/mL	5	2.5	0.5
#1	+	+	+	+		-	-	-	-
#2	+	+	+	+		-	-	-	-
#3	+	+	+	+		-	-	-	-
	R2A sterile	**ISP2 sterile control**	**V8 sterile control**	**Sterile TSB**	**2% Virkon**	**0.1 N HCl**			
1#	**-**	**-**	**-**	**+**	**-**	**-**			
#2	**-**	**-**	**-**	**+**	**-**	**-**			
#3	**-**	**-**	**-**	**+**	**-**	**-**			

Day 8 Biofilm recovery growth check (+=Growth, -= No growth, RED= reduced growth)

Non	A1A3 R2A NON	A1A3 ISP2 NON	A1A3 V8 NON	R003 R2A NON	R003 ISP2 NON	R003 V8 NON	58B R2A NON	58B ISP2 NON	58B V8NON
#1	+	+	**RED**	**RED**	**RED**	**RED**	**-**	+	**RED**
#2	+	+	+	+	+	+	+	+	+
#3	+	+	+	+	+	**RED**	+	+	+
UV	A1A3 R2A	A1A3 ISP2	A1A3 V8	R003 R2A	R003 ISP2	R003 V8	58B R2A	58B ISP2	58B V8
#1	+	+	+	+	+	+	+	+	+
#2	+	+	+	+	+	+	+	+	**-**

#3	+	+	+	+	+	**RED**	+	+	-
Antibiotic	**Tetra HCl 120 ug/mL**	60	30	10		**Cipro** 10 ug/mL	5	2.5	0.5
#1	+	+	+	+		-	-	-	-
#2	+	+	+	+		-	-	-	-
#3	+	+	+	+		-	-	-	-
	R2A sterile	**ISP2 sterile control**	**V8 sterile control**	**Sterile TSB**	**2% Virkon**	**0.1 N HCl**			
1#	-	-	-	+	-	-			
#2	-	-	-	+	-	-			
#3	-	-	-	+	-	-			

Day 10 Biofilm recovery growth check (+=Growth, -= No growth, RED= reduced growth)

Non	A1A3 R2A NON	A1A3 ISP2 NON	A1A3 V8 NON	R003 R2A NON	R003 ISP2 NON	R003 V8 NON	58B R2A NON	58B ISP2 NON	58B V8NON
#1	+	+	+	+	+	+	+	+	+
#2	+	+	+	+	+	+	+	+	+
#3	+	+	+	+	+	+	+	+	+
UV	A1A3 R2A	A1A3 ISP2	A1A3 V8	R003 R2A	R003 ISP2	R003 V8	58B R2A	58B ISP2	58B V8
#1	+	+	+	+	+	+	+	+	+
#2	+	+	+	+	+	+	+	+	+
#3	+	+	+	+	+	+	+	+	+
Antibiotic	**Tetra HCl 120 ug/mL**	60	30	10		**Cipro** 10 ug/mL	5	2.5	0.5

#1	+	+	+	+		-	-	**+**	**+**
#2	+	+	+	+		-	-	**+**	**+**
#3	+	+	+	+		-	-	**+**	**+**
	R2A sterile	**ISP2 sterile control**	**V8 sterile control**	**Sterile TSB**	**2% Virkon**	**sterile water**			
1#	**-**	**-**	**-**	**+**	**-**	**+**			
#2	**-**	**-**	**-**	**+**	**-**	**+**			
#3	**-**	**-**	**-**	**+**	**-**	**+**			

Day 2 KB disc diffusion(+=Inhibition, -= NO inhibition, #= diameter of inhibitory zone in mm)

Non	A1A3 R2A NON	A1A3 ISP2 NON	A1A3 V8 NON	R003 R2A NON	R003 ISP2 NON	R003 V8 NON	58B R2A NON	58B ISP2 NON	58B V8NON
#1	-	-	-	-	-	-	-	-	-
#2	-	-	-	-	-	-	-	-	-
#3	-	-	-	-	-	-	-	-	-
UV	A1A3 R2A	A1A3 ISP2	A1A3 V8	R003 R2A	R003 ISP2	R003 V8	58B R2A	58B ISP2	58B V8
#1	-	-	-	-	-	-	-	-	-
#2	-	-	-	-	-	-	-	-	-
#3	-	-	-	-	-	-	-	-	-
Antibiotic	**Tetra HCl 120 ug/mL**	60	30	10		**Cipro** 10 ug/mL	5	2.5	0.5
#1	10	-	-	-		26	18	12	-
#2	+	-	-	-		25	16	10	-

#3	+	-	-	-		24	18	10	-
	R2A sterile	**ISP2 sterile control**	**V8 sterile control**	**Sterile TSB**	**2% Virkon**	**0.1 N HCl**			
1#	**-**	**-**	**-**	**-**	**+**	**-**			
#2	**-**	**-**	**-**	**-**	**+**	**-**			
#3	**-**	**-**	**-**	**-**	**+**	**-**			

Day 4 KB disc diffusion(+=Inhibition, -= NO inhibition, #= diameter of inhibitory zone in mm)

Non	A1A3 R2A NON	A1A3 ISP2 NON	A1A3 V8 NON	R003 R2A NON	R003 ISP2 NON	R003 V8 NON	58B R2A NON	58B ISP2 NON	58B V8NON
#1	-	-	-	-	-	-	-	-	-
#2	-	-	-	-	-	-	-	-	-
#3	-	-	-	-	-	-	-	-	-
UV	A1A3 R2A	A1A3 ISP2	A1A3 V8	R003 R2A	R003 ISP2	R003 V8	58B R2A	58B ISP2	58B V8
#1	-	-	-	-	-	-	-	-	-
#2	-	-	-	-	-	-	-	-	-
#3	-	-	-	-	-	-	-	-	-
Antibiotic	**Tetra HCl 120 ug/mL**	60	30	10		**Cipro** 10 ug/mL	5	2.5	0.5
#1	14	-	-	-		23	15	+	-
#2	14	-	-	-		21	17	+	-
#3	13	-	-	-		20	15	+	-
	R2A sterile	**ISP2 sterile**	**V8 sterile**	**Sterile TSB**	**2% Virkon**	**0.1 N HCl**			

		control	**control**						
1#	**-**	**-**	**-**	**-**	**+**	**-**			
#2	**-**	**-**	**-**	**-**	**+**	**-**			
#3	**-**	**-**	**-**	**-**	**+**	**-**			

Day 6 KB disc diffusion(+=Inhibition, -= NO inhibition, #= diameter of inhibitory zone in mm)

Non	A1A3 R2A NON	A1A3 ISP2 NON	A1A3 V8 NON	R003 R2A NON	R003 ISP2 NON	R003 V8 NON	58B R2A NON	58B ISP2 NON	58B V8NON
#1	-	-	-	-	-	-	-	-	-
#2	-	-	-	-	-	-	-	-	-
#3	-	-	-	-	-	-	-	-	-
UV	A1A3 R2A	A1A3 ISP2	A1A3 V8	R003 R2A	R003 ISP2	R003 V8	58B R2A	58B ISP2	58B V8
#1	-	-	-	-	-	-	-	-	-
#2	-	-	-	-	-	-	-	-	-
#3	-	-	-	-	-	-	-	-	-
Antibiotic	**Tetra HCl 120 ug/mL**	60	30	10		**Cipro** 10 ug/mL	5	2.5	0.5
#1	+	-	-	-		23	16	+	-
#2	+	-	-	-		20	15	+	-
#3	+	-	-	-		21	12	+	-
	R2A sterile	**ISP2 sterile control**	**V8 sterile control**	**Sterile TSB**	**2% Virkon**	**0.1 N HCl**			

1#	**-**	**-**	**-**	**-**	**+**	**-**			
#2	**-**	**-**	**-**	**-**	**+**	**-**			
#3	**-**	**-**	**-**	**-**	**+**	**-**			

Day 8 KB disc diffusion (+=Inhibition, -= NO inhibition, #= diameter of inhibitory zone in mm)

Non	A1A3 R2A NON	A1A3 ISP2 NON	A1A3 V8 NON	R003 R2A NON	R003 ISP2 NON	R003 V8 NON	58B R2A NON	58B ISP2 NON	58B V8NON
#1	-	-	-	-	-	-	-	-	-
#2	-	-	-	-	-	-	-	-	-
#3	-	-	-	-	-	-	-	-	-
UV	A1A3 R2A	A1A3 ISP2	A1A3 V8	R003 R2A	R003 ISP2	R003 V8	58B R2A	58B ISP2	58B V8
#1	-	-	-	-	-	-	-	-	-
#2	-	-	-	-	-	-	-	-	-
#3	-	-	-	-	-	-	-	-	-
Antibiotic	**Tetra HCl 120 ug/mL**	60	30	10		**Cipro** 10 ug/mL	5	2.5	0.5
#1	10	+	-	-		20	14	+	-
#2	12	+	-	-		20	11	+	-
#3	11	+	-	-		21	12	+	-
	R2A sterile	**ISP2 sterile control**	**V8 sterile control**	**Sterile TSB**	**2% Virkon**	**0.1 N HCl**			
1#	**-**	**-**	**-**	**-**	**+**	**-**			

#2	-	-	-	-	+	-			
#3	-	-	-	-	+	-			

Day 10 KB disc diffusion (+=Inhibition, -= NO inhibition, #= diameter of inhibitory zone in mm)

Non	A1A3 R2A NON	A1A3 ISP2 NON	A1A3 V8 NON	R003 R2A NON	R003 ISP2 NON	R003 V8 NON	58B R2A NON	58B ISP2 NON	58B V8NON
#1	-	-	-	-	-	-	-	-	-
#2	-	-	-	-	-	-	-	-	-
#3	-	-	-	-	-	-	-	-	-
UV	A1A3 R2A	A1A3 ISP2	A1A3 V8	R003 R2A	R003 ISP2	R003 V8	58B R2A	58B ISP2	58B V8
#1	-	-	-	-	-	-	-	-	-
#2	-	-	-	-	-	-	-	-	-
#3	-	-	-	-	-	-	-	-	-
Antibiotic	**Tetra HCl 120 ug/mL**	60	30	10		**Cipro** 10 ug/mL	5	2.5	0.5
#1	+	+	-	-		19	11	+	-
#2	+	-	-	-		19	10	+	-
#3	+	-	-	-		20	11	+	-
	R2A sterile	**ISP2 sterile control**	**V8 sterile control**	**Sterile TSB**	**2% Virkon**	**Water sterile control**			
1#	**-**	**-**	**-**	**-**	**+**	**-**			

#2	-	-	-	-	+	-			
#3	-	-	-	-	+	-			

APPENDIX C-CONTIGUOUS SEQUENCES OF BACTERIA

> Contig - A1A3_518-800
GGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGCCTTTCGGGGTGGATTAGTGGC
GAACGGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATAC
CGGATAACACTCTGTCCTGCATGGGACGGGGTTAAAAGCTCCGGCGGTGAAGGATGAGCCCGCGGCCTAT
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